Functional Analysis of the Profilaggrin N-Terminal Peptide: Identification of Domains that Regulate Nuclear and Cytoplasmic Distribution

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Profilaggrin is expressed in the differentiating granular layer of epidermis and other stratified epithelia, where it forms a major component of cytoplasmic keratohyalin granules. It consists of two distinct domains, an N-terminal S100-like Ca^{2+}- binding domain containing two EF-hands and multiple filaggrin units that aggregate keratin filaments in the stratum corneum. Here, we report structure–function studies of the N-terminal peptide from mouse, human, and rat profilaggrin. The profilaggrin N-terminal peptides of all species contain two S100-like EF-hands, bipartite nuclear localization sequences, and proprotein convertase cleavage sites. The nuclear localization signals in human and mouse profilaggrin were shown to be functional by transfection of epithelial cells and depended on the absence of filaggrin sequences. The nuclear localization of the processed (free) N-terminal peptide of human profilaggrin is consistent with immunolocalization findings in normal human skin and in parakeratotic skin disorders, which exhibit nuclear staining of granular and/or cornified layers. The mouse profilaggrin N-terminus undergoes proteolytic processing in two steps, first releasing an N-terminal peptide containing some filaggrin sequence and finally the free N-terminus of 28–30 kDa; these peptides have cytoplasmic and nuclear distributions, respectively, when expressed in transfected cells. The N-terminal processing may occur prior to or simultaneously with the proteolytic processing of the polyfilaggrin domain. The nuclear accumulation of the profilaggrin N-terminal peptide in epidermis and in transfected cells strongly suggests a calcium-dependent nuclear function for the profilaggrin N-terminus during epidermal terminal differentiation when the free N-terminus is released from profilaggrin by specific proteolysis. ***Key words: epidermal differentiation/keratinocyte/nuclear localization/profilaggrin/proteolytic processing/transfection. J Invest Dermatol 119:661–669, 2002***

The terminal differentiation of epidermal keratinocytes results in the formation of a mechanically resistant and toughened structure, the stratum corneum, which functions to protect mammals against dessication, and physical and chemical damage. Among the many morphologic changes that occur in normal epidermis during the transition from granular to cornified cells are the dissolution of the nucleus and other organelles, the aggregation of the keratin intermediate filament (IF) network into macrofibrils, and the formation of a cornified cell envelope consisting of loricrin, the small proline-rich proteins, involucrin, and other proteins that are crosslinked by transglutaminases in the stratum corneum (Nemes and Steinert, 1999; Presland and Dale, 2000). Although the mechanism of nuclear destruction is not understood, the cross-linking and collapse of keratin IFs into macrofibril bundles is mediated by the IF-associated protein filaggrin, an abundant protein present in granular and transition cells produced by the specific proteolysis of profilaggrin.

Profilaggrin is a large, highly phosphorylated, insoluble protein consisting of multiple (10–12 in human, 20 or more in rodents) filaggrin units joined by linker peptides. Profilaggrin is expressed in the granular layer where it is localized within electron-dense nonmembrane bound keratohyalin granules (reviewed in Dale *et al*, 1994; Presland and Dale, 2000). This sequestration into keratohyalin is believed to protect the epithelial cell from the deleterious effects of premature keratin aggregation by filaggrin (Dale *et al*, 1997; Kuechle *et al*, 1999; Presland *et al*, 2001).

The profilaggrin N-terminus consists of two distinct domains: a conserved A domain that contains two Ca^{2+}- binding S100-like EF-hands that each bind a Ca^{2+} ion, and a less conserved cationic B domain (Presland *et al*, 1992, 1995; Markova *et al*, 1993). Profilaggrin belongs to a growing family of high molecular weight proteins with N-terminal S100 domains fused to structurally distinct repetitive regions that are proposed to function as IF-associated proteins and/or cornified envelope components (Fietz *et al*, 1993; Lee *et al*, 1993; Krieg *et al*, 1997; Makino *et al*, 2001). The genes encoding these "fused S100" proteins are among a large

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Abbreviations: bNLS, bipartite nuclear localization sequence; EDC, epidermal differentiation complex; GFP, green fluorescent protein; IF, intermediate filament; MEKs, mouse epidermal keratinocytes; PC, proprotein convertase; REKs, rat epidermal keratinocytes; TBS, Tris-buffered saline.

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number of epidermally expressed genes that map to human chromosome 1q21 in an ≈1.5 mB region termed the epidermal differentiation complex (EDC) (Marenholz *et al*, 1996; Mischke *et al*, 1996; South *et al*, 1999). An equivalent EDC is present on mouse chromosome 3 (Song *et al*, 1999).

During terminal differentiation profilaggrin is processed by several endoproteases to generate two major products: filaggrin and the N-terminal peptide. Profilaggrin processing to filaggrin requires both the removal of multiple phosphates from each filaggrin unit within profilaggrin by phosphatases and its specific proteolysis by a number of proteases (Lonsdale-Eccles *et al*, 1982; Harding and Scott, 1983; Resing *et al*, 1989; Kam *et al*, 1993). The proteases that are involved in profilaggrin processing include profilaggrin endoproteinase 1 (PEP1), a chymotrypsin-like protease (Resing *et al*, 1995b), calpain (Resing *et al*, 1993a; Yamazaki *et al*, 1997), and furin, a member of the proprotein convertase (PC) family of endoproteases (Pearton *et al*, 2001). The order of these processing events has not been elucidated; however, as profilaggrin intermediates containing two or more filaggrin units lack detectable phosphate it is thought that most dephosphorylation occurs before processing of the polyfilaggrin domain occurs (Harding and Scott, 1983).

Filaggrin binds to cytoplasmic keratin IFs to form the macrofibrils that are retained in cornified cells, whereas the N-terminal peptide localizes to nuclei of epidermal granular and transition cells (Ishida-Yamamoto *et al*, 1998; Presland and Dale, 2000). The mechanism and function of this nuclear translocation is unclear. In loricrin keratoderma, a disease affecting palm and sole skin characterized by parakeratosis in the lower cornified layers, the profilaggrin N-terminus is associated with nuclear aggregates of a mutant loricrin protein that might prevent it from functioning normally (Ishida-Yamamoto and Iizuka, 1998; Ishida-Yamamoto *et al*, 1998). Studies of affected patients and transgenic mice that are either loricrin-deficient and/or express the same mutant protein have shown that the loricrin mutation is pathogenic, and that the resulting skin disease is probably due to disruption of nuclear events during terminal differentiation rather than to an absence of loricrin from cornified envelopes (Ishida-Yamamoto *et al*, 2000; Koch *et al*, 2000; Suga *et al*, 2000).

In this paper, we report the sequence of the mouse and rat profilaggrin N-termini and show its similarity to the human profilaggrin N-terminus in terms of both structure and proteolytic processing in keratinocytes. Like its human counterpart, the mouse N-terminal peptide is specifically cleaved *in vivo* during terminal differentiation. We show that the cationic B domain of the profilaggrin N-terminus contains functional bipartite nuclear localization sequence(s) (bNLSs) that facilitate nuclear accumulation in keratinocytes. Removal of filaggrin sequences by proteolytic cleavage, however, is required for this nuclear translocation. Profilaggrin processing therefore provides a potential link between two major events of terminal differentiation: the aggregation of keratin filaments and nuclear dissolution.

MATERIALS AND METHODS

Isolation and characterization of mouse and rat profilaggrin genomic and cDNA clones A 129/SvJ mouse genomic BAC library (Genome Systems, St. Louis, MO) was screened with a pair of polymerase chain reaction (PCR) primers specific for the mouse profilaggrin 3′ noncoding sequence. BAC plasmid DNA was isolated from overnight cultures of each clone using Nucleobond AX columns (The Nest Group, Southboro, MA). The profilaggrin gene was localized within BAC clones by southern blot hybridization of restriction digests with coding and 3′ noncoding filaggrin DNA probes labeled with [α-^{32}P]-dCTP (3000 Ci per mmol) (Sambrook *et al*, 1989). Several restriction endonuclease fragments that hybridized with coding probes were subcloned into pGEM5Zf+ and pGEM7Zf+ vectors (Promega, Madison, WI) and sequenced to derive the 5′ end of the mouse profilaggrin coding sequence. To confirm the coding sequence and exon/intron organization of the mouse profilaggrin gene, the cDNA sequence was isolated by reverse transcription PCR (RT-PCR) of mouse epidermal RNA as described previously (Presland *et al*, 2000) using the following primers derived from the genomic sequence: 5′-GTGCATACACACTACTA (upstream exon 2 primer) and 5′-AGAAAGTAGTAGGCATG (downstream exon 3 primer). The cDNA product (≈1 kb) containing the N-terminal domain of mouse profilaggrin was cloned into TOPO-TA vector (Invitrogen, Carlsbad, CA) for sequence analysis.

For rat profilaggrin, genomic and cDNA sequences were obtained by PCR amplification of DNA and RNA isolated from rat epidermal keratinocytes (REKs) (Baden and Kubilus, 1983; Haydock *et al*, 1993). Genomic DNA was isolated using a Qiamp tissue kit (Qiagen, Chatsworth, CA). For genomic DNA amplifications, PCR assays (32 cycles at 94°C for 50 s, 47.5°C for 60 s, and 72°C for 90 s) were performed using Taq polymerase (Promega, Madison, WI) in the presence of 5% dimethylsulfoxide and 2.5 mM $MgCl_2$. The 5′ primer corresponded to nucleotides 1–24 of the coding region of the human profilaggrin sequence (5′-ATGTCTACTCTCCTGGAAAACATC-3′; GenBank accession L01088). The 3′ primer was directed against the linker region of the rat filaggrin sequence (5′-GGACTC-GTCCTCGGTTTCTTCTACACC-3′; GenBank accession M21759). The resulting 2.2 kb PCR products were cloned into pCR2.1 TA (Invitrogen) for sequencing. Sequence analysis of both mouse and rat profilaggrin clones was performed by dye terminator cycle sequencing using the BigDye kit (Perkin Elmer ABI, Foster City, CA). To obtain the entire sequence for both strands of mouse and rat profilaggrin, internal primers were synthesized and the resultant overlapping sequences were assembled using either the Sequencher program (Gene Codes, Ann Arbor, MI) or PC/GENE.

To determine the exon/intron boundaries of the rat profilaggrin sequence, total RNA was extracted from confluent REKs and single stranded cDNA was prepared as described previously (Presland *et al*, 2001). PCR was performed with the primers 5′-CACTAG-CATGATTGA CATATTCC-3′ and 5′-TTGAAGTCTGCCCTT-GCCCG-3′, using 32 cycles of 94°C for 50 s, 53°C for 45 s, and 72°C for 50 s in the presence of Taq polymerase (Promega) and Taq extender (Stratagene, La Jolla, CA). The PCR product (1078 bp) was cloned into pCR2.1 (Invitrogen) and sequenced. A partial genomic sequence was also obtained from Sprague-Dawley rats that express a small 350 kDa variant of profilaggrin containing 7–9 filaggrin units (K. Resing, personal communication) using the 5′ primer CATGCAAATGCTAGATGTGG-3′ and the same 3′ primer used above.

The N-terminal sequence of rat profilaggrin protein was verified by Endolys C digestion of purified rat profilaggrin followed by matrix assisted laser desorption ionization mass spectrometry and electrospray ionization mass spectrometry (Resing *et al*, 1993b; 1995a) to be STLLESITSMIDIFQQYSNNDK (K. Resing, personal communication). This sequence exhibits four amino acid differences from mouse and nine from human profilaggrin (see **Fig 1*B***).

Sequence alignments were performed using the programs NALIGN (PC/GENE) and CLUSTALW (PBIL) and outputted with BOXSHADE. Peptide motifs were identified using the programs PROSITE (http://c.expasy.org/prosite) and PSORT II (http://psort.-nibb.ac.jp).

Production of an antibody against the mouse profilaggrin N-terminus A polyclonal antipeptide antibody, Am1, was developed against a 15 amino acid sequence (E_{38}GQLQAVLKNPDDQD$_{53}$) in the region between the EF-hands of the mouse A domain (see **Fig 1*B***) (Genemed Synthesis, San Francisco, CA). This sequence is equivalent to the peptide used to prepare the human-A-domain-specific antibody (Presland *et al*, 1997). The antibodies were affinity purified prior to use.

Immunoblotting of mouse and human extracts Cell or tissue extracts were prepared in urea–Tris buffer as previously described (Presland *et al*, 1997), or in a modified RIPA buffer containing 1% Nonidet P-40 (Pearton *et al*, 2001). For immunoblotting, equal protein loadings were resolved on either 7%–12% or 10%–15% gradient sodium dodecyl sulfate (SDS) polyacrylamide gels and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). After blocking with 2% nonfat dry milk powder the membranes were probed with primary antibody and detected with a horseradish-peroxidase-conjugated donkey antirabbit IgG (Amersham, Arlington Heights, IL) using the Renaissance chemiluminescent substrate (NEN, Boston, MA).

Mouse keratinocyte culture Mouse epidermal keratinocytes (MEKs) were cultured in low (0.06 mM) Ca^{2+} NIH-3T3 fibroblast conditioned medium as described previously (Hager *et al*, 1999). Cells were passaged and seeded at low density (2.4×10^4 cells per cm^2) and reached confluence in 7 d. Confluent cultures were fed with 3T3-conditioned

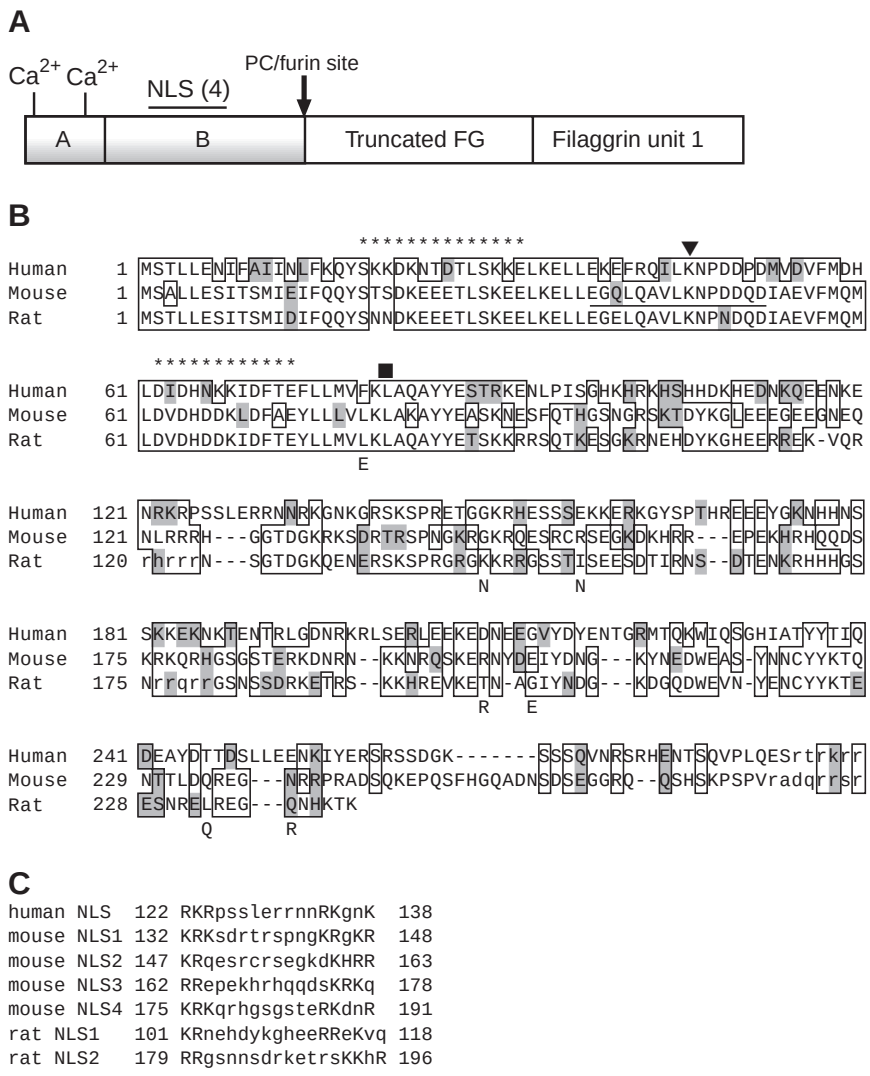

Figure 1. Structure of mouse profilaggrin and conservation of functional elements among human and rodent profilaggrins. (*A*) Structure of mouse profilaggrin. Mouse profilaggrin consists of an N-terminal A and B domain (283 amino acids) followed by a truncated filaggrin unit of 173 residues and ≈20 filaggrin units. Only the first filaggrin unit of 250 amino acids is shown. Like other mammalian profilaggrins, the C-terminal end of the protein contains a truncated filaggrin unit and unique peptide of 23 amino acids (Rothnagel *et al*, 1987; GenBank Accession Number J03458). The protein motifs located in the mouse A and B domains are indicated. (*B*) Predicted amino acid sequences of human, mouse, and rat profilaggrin N-termini. The S100Ca^{2+}-binding domain is highly conserved (amino acids 1–80) whereas the B domain is less conserved. Indicated are the two S100EF-hands (*stars*) and the furin/PC cleavage sites (*lower case letters*). In human and mouse profilaggrin, the single PC cleavage site is located at the end of the N-terminal sequence, whereas in rat profilaggrin they are at amino acids 120–124 and 176–180. The sequence used to generate the Am1 antibody specific for mouse profilaggrin (amino acids 38–53) is *underlined*. The *arrowhead* at amino acid 47 indicates the conserved location of the intron between the two S100-like EF-hands; this intron is 1670 bp and 681 bp in the mouse and rat profilaggrin genes, respectively. The *solid box* indicates the end of the A domain at residue 80. Boxed regions indicate amino acid identity between sequences and light shading indicates similarity. Residues shown below the rat sequence indicate amino acid differences seen in the 350 kDa profilaggrin variant partially sequenced from Sprague-Dawley rats. (*C*) Amino acid sequences of the bipartite nuclear localization signals in human, mouse, and rat profilaggrin N-terminus. The basic amino acids in each sequence are shown *capitalized*. The profilaggrin sequences reported here have been deposited to Genbank with accession numbers of AF510859 and AF510860 (mouse profilaggrin) and AY102923 (rat profilaggrin).

medium every 48 h and the detached cells were harvested by centrifugation of the culture medium. The cell pellet was washed with cold Dulbecco's phosphate-buffered saline (PBS) before extraction. Adherent cells were washed twice with cold PBS before being scraped from the dish and extracted in urea–Tris or modified RIPA buffer. Unextracted cell pellets and cell extracts were stored at –80°C until use. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) and equal protein loads from each pellet were loaded onto SDS polyacrylamide gels and blotted onto nitrocellulose for Western blot detection.

The phosphorylation state of profilaggrin and its processing products was determined by labeling confluent keratinocytes with radiolabeled phosphate. Two day postconfluent MEKs were grown in the presence of 100 μCi [^{32}P]-orthophosphate for 24 h. Labeled cells were harvested by centrifugation of the medium (for detached cells) or scraping the dish surface (for attached cells). After washing away unbound orthophosphate in cold PBS, cells were extracted in urea–Tris or RIPA buffer. The RIPA buffer extract was subjected to immunoprecipitation using either antimouse profilaggrin antibody (Covance, Richmond, CA) or the Am1 antibody. Antibody–antigen complexes were recovered using protein-A-coated paramagnetic beads (Dynal, Lake Pleasant, NY), eluted with 0.1 M glycine pH 2.8 or by heating at 45°C for 15 min in 2% SDS Laemmli loading buffer. The extracts and immunoprecipitates were run on 7.5%–12% SDS polyacrylamide gels and either blotted onto nitrocellulose for Western analysis or dried and exposed to X-ray film.

Immunofluorescence microscopy Immunofluorescence was performed on frozen or methyl Carnoys fixed tissue samples embedded in paraffin (Presland *et al*, 1997) or transfected cells grown on glass coverslips fixed with ice-cold methanol:acetone (3:1) (Dale *et al*, 1997). After rehydration (for Carnoys fixed tissues) sections were washed in Tris-buffered saline (TBS) and processed for single or double immunofluorescence. Sections were incubated with primary antibody overnight at 4°C in TBS containing 1% bovine serum albumin. The primary antibodies were detected using either fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG (Vector Laboratories, Burlingame, CA) or biotinylated goat antirabbit IgG followed by streptavidin conjugated to Texas Red (Vector Laboratories). For detection of mouse filaggrin a directly labeled FITC-antifilaggrin rabbit IgG (Covance) was used after the initial secondary was removed. Multiple rinses in TBS were done between each step. For DNA staining, samples were incubated in 0.001% 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, St. Louis, MO) for 10 min and then washed with water. Samples were air-dried and coverslipped using Prolong mounting medium (Molecular Probes, Eugene, OR) to minimize fading. All the samples were observed under an epifluorescence Nikon-SA Microphot microscope and digital images were collected using a CCD camera (Photometrics, Tucson, AZ).

Constructs and transfection procedures DNA constructs were prepared from mouse and human profilaggrin cDNA clones (Presland *et al*, 1997; this paper) by a PCR approach followed by cloning into NT-GFP-TOPO (Invitrogen) to generate constructs with an N-terminal green fluorescent protein (GFP) tag. The primers used for cloning the complete mouse profilaggrin N-terminus were 5′-TCCGCTCTC-CTGGAAAGCATC-3′ (MP-5) and 5′-TTAGCCTGCCCTGGATCT-CCTCTG-3′ (MP-3); the human primers were 5′-TCTACTCTC-CTGGAAAACATCTTTG-3′ (HP-5) and 5′-TTACCTACGCTTTCT-

TGTCCTGG-3′ (HP-3) (termination codons engineered at the 3′ end of each construct are underlined). The human profilaggrin N-terminal mutant and deletion constructs were prepared using a similar approach (see **Fig 6**; primer sequences are available upon request). Deletion constructs of the mouse profilaggrin N-terminus fused to GFP were prepared in a similar manner using the common upstream primer MP-5 in combination with the following downstream primers: MP192–GFP, 5′-TTAATTTCTGTTGTCCTTTCTTTCG-3′; MP174–GFP, 5′-TTATGAATCTTGTTGGTGTCTGTG-3′; MP161–GFP, 5′-TTAATGTTTATCTTTTCCCTCACTTC-3′; MP145–GFP, TTATCTTTTTCCATTTGGGCTTCTCG-3′; and MP131–GFP, 5′-TTATCCATCAGTTCCACCATGCCTC-3′. All downstream primers contain termination codons at the end of the encoded profilaggrin peptide (underlined). All constructs were verified by DNA sequencing.

COS-7 cells were transfected with DNA constructs at 60%–70% of confluence using Lipofectamine reagent (Invitrogen) as described previously (Dale *et al*, 1997). Cells were harvested or fixed for staining 48 or 72 h post-transfection. REKs [a gift of H. Baden, Massachusetts General Hospital and Cutaneous Biology Research Center, Boston, MA (Baden and Kubilus, 1983)] were transfected as described previously (Dale *et al*, 1997). GFP epifluorescence and antibody labeling of transfected cells with N-terminal antibodies were carried out as described previously (Dale *et al*, 1997; Kuechle *et al*, 1999).

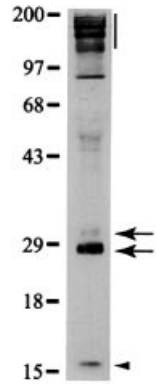

Figure 2. N-terminal processing of profilaggrin in mouse epidermis. A mouse epidermal urea–Tris extract was run on a 7%–12% SDS polyacrylamide gel, blotted to nitrocellulose membrane, and reacted with the Am1 antibody directed against the mouse A domain. The antibody recognizes profilaggrin and potential large processing products (*bar*) as well as bands at ≈90 kDa, a doublet at 28–30 kDa (*arrows*), and a band at 16 kDa (*arrowhead*).

RESULTS

Characterization of the mouse and rat profilaggrin N-termini and identification of putative functional motifs Mouse and rat profilaggrin genomic and cDNA clones were obtained by a combination of approaches (see *Materials and Methods*), and clones were sequenced to derive the corresponding N-terminal protein sequences. The mouse and rat profilaggrin N-terminal peptides are 283 and 242 amino acids in length, respectively, including the initiator methionine. The A domain that contains the S100-like EF-hands is highly conserved between human, mouse, and rat profilaggrin (56.8% identity, 72.8% similarity; **Fig 1*B***). Other features shared are PC/furin-like cleavage sites located at the end of the unique nonfilaggrin N-terminal region (human, mouse) or within the B domain (rat), and the presence of bNLSs in the cationic B domain (**Fig 1*C***) that conform to the consensus described for this motif (Dingwall and Laskey, 1986). Overall the N-termini of human, mouse, and rat profilaggrin show 28.9% identity and 43.4% similarity. As expected from evolutionary considerations, the mouse profilaggrin N-terminal sequence exhibits a greater similarity to rat than to human (58.3% *vs* 38% identity, respectively); this is most readily apparent in the B domain (**Fig 1*B***). The nucleic acid and predicted amino acid sequences of the mouse profilaggrin N-terminus obtained from 129/SvJ (BAC clone) and C57BL/6J (cDNA sequence) mice were identical, whereas there were seven amino acid differences between the two rat profilaggrin sequences examined (**Fig 1*B***).

Mouse profilaggrin undergoes N-terminal processing during epidermal differentiation A polyclonal antibody, Am1, was produced against a sequence in the mouse A domain (E_{38}GQLQAVLKNPDDQD_{53}) (**Fig 1*B***). This sequence has a high Smith–Waterman antigenicity index and is exposed on the surface in crystal structures of other S100 proteins such as S100B and S100A7 (Kilby *et al*, 1996; Brodersen *et al*, 1998; Sastry *et al*, 1998; Smith and Shaw, 1998). On Western blots, the mouse N-terminal antibody Am1 recognized high molecular weight mouse profilaggrin, a 90 kDa band that probably represents an intermediate in profilaggrin processing, and a series of smaller polypeptides consisting of a doublet at 28–30 kDa and a band at ≈16 kDa (**Fig 2**). The 28–30 kDa doublet corresponds closely in size to the predicted molecular weight of the mouse profilaggrin N-terminus (282 residues, 31 kDa) demonstrating that the mouse profilaggrin N-terminus undergoes N-terminal processing as has been described for the human protein (Presland *et al*, 1997). The presence of more than one N-terminal peptide may represent additional proteolytic trimming or phosphorylation.

The Am1 antibody showed a stronger and cleaner reaction in the presence of 5 mM ethylenediamine tetraacetic acid (data not shown). This Ca^{2+} dependence of the Am1 reaction can be explained by the position of the antigen (residues 38–53) between the two Ca^{2+}-binding EF-hands in the A domain (**Fig 1*B***). The binding of Ca^{2+} to the profilaggrin A domain apparently abrogates antibody binding either by steric hindrance or by a conformation change in the protein, as demonstrated for the human N-terminal peptide (Presland *et al*, 1995).

The lower molecular weight peptides of 28–30 kDa and 16 kDa were present in soluble (nonurea) extracts of mouse skin isolated in both the presence and absence of detergents (data not shown) although reaction with peptides of < 20 kDa was variable and depended on protein load, extraction, and blotting conditions (compare **Figs 2** and **4*A***). These peptide(s) may represent either further processing of the mouse profilaggrin N-terminus or crossreaction with other S100 proteins (Presland *et al*, 1997).

The mouse A domain antibody showed no reactivity towards rat profilaggrin or processing intermediates, even though the peptide sequence used to generate the antibody was 87% (13 out of 15 residues) identical with the analogous sequence from rat profilaggrin (**Fig 1*B***).

The mouse profilaggrin N-terminus localizes to keratohyalin granules in epidermal granular cells The Am1 antibody showed strong immunolabeling of keratohyalin granules in the epidermal granular layer (**Fig 3*B–D***) where it colocalized with the filaggrin antibody immunolocalization (**Fig 3*A*, *C***). The Am1 staining pattern does not colocalize with filaggrin in the stratum corneum, however, suggesting a different fate for this peptide during terminal differentiation.

The profilaggrin N-terminus undergoes partial processing *in vitro* Mouse profilaggrin undergoes N-terminal processing in epidermis to release a peptide that corresponds closely in size to the free mouse N-terminus (**Fig 2**). To examine profilaggrin processing *in vitro*, MEKs were grown to confluence under low Ca^{2+} conditions in which the detached cells have been shown previously to express markers of differentiation such as profilaggrin and K1, and to partially process profilaggrin (Missero *et al*, 1995; Di Cunto *et al*, 1998). MEKs grown under these conditions expressed profilaggrin (> 200 kDa) and a protein of 50 kDa that reacted with both Am1 and filaggrin antibodies (*bar* and *arrows*, respectively, **Fig 4*A***). The 50 kDa peptide was also detected with filaggrin antibody following immunoprecipitation of MEK proteins with the profilaggrin Am1 antibody (**Fig 4*B***, *arrow*). Because the 50 kDa

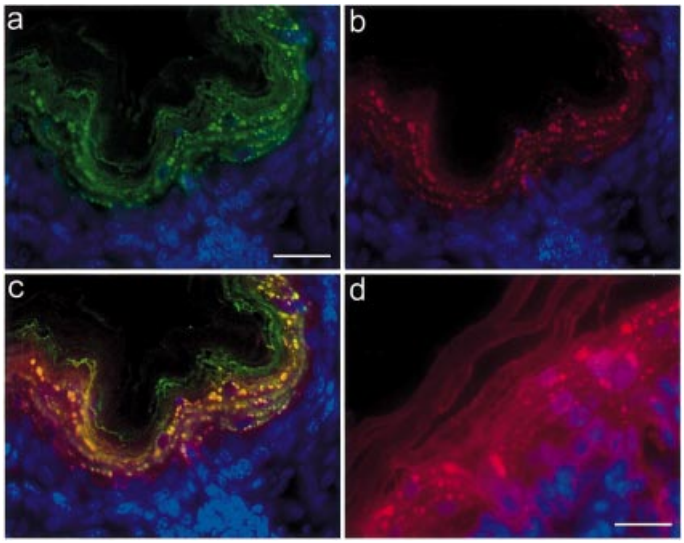

Figure 3. Immunofluorescence localization of mouse profilaggrin N-terminal and filaggrin antibodies in newborn skin. Frozen mouse skin was stained with antibodies against mouse filaggrin (*A*) and the N-terminal (Am1) antibody (*B*, *D*). Part (*C*)is a merged image of (*A*) and (*B*). Both filaggrin and the Am1 antibody stain keratohyalin granules in the granular layer (*A*, *B*, yellow in *C*) whereas in the cornified and transition layers the immunostaining no longer colocalizes. The filaggrin antibody stains the lower cornified layers very strongly but diminishes in the upper layers due to the degradation of filaggrin to amino acids. *Scale bars*: (*A*)–(*C*) 50 μm; (*D*) 25 μm.

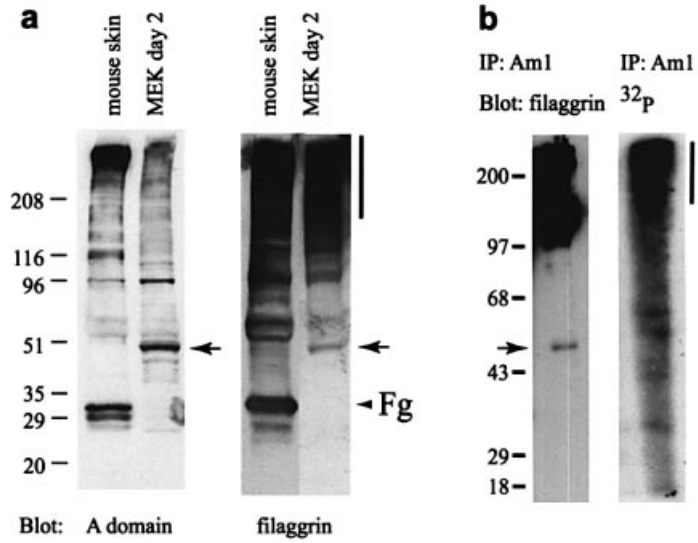

Figure 4. Analysis of profilaggrin processing *in vitro*. (*A*) Detached MEKs were harvested at 2 d after confluence, extracted in urea–Tris buffer, and run on 7%–12% SDS polyacrylamide gels for Western blotting. Duplicate blots were probed with antibodies against the mouse N-terminus (A domain, Am1) and filaggrin. Detached cells produced full-length profilaggrin (*bar*), as well as a 50 kDa product that reacted with both the N-terminus and filaggrin antibodies (*arrows*). Cultured MEKs did not fully process profilaggrin to the 28–30 kDa doublet (*left panel*) or mature filaggrin (Fg, *right panel*) present in mouse epidermis. (*B*) MEK cultures were grown in the presence of 100 μCi of inorganic ^{32}P. After 24 h the detached cells were harvested and extracted in RIPA buffer and immunoprecipitated (IP) with the Am1 antibody. The immunoprecipitated proteins were run on 10%–15% SDS polyacrylamide gels and either blotted and probed with a filaggrin antibody (*left panel*) or dried down and exposed to X-ray film (*right panel*). The Am1 antibody precipitated a 50 kDa protein that reacted with the filaggrin antibody (*arrow*) but no corresponding ^{32}P band was visible in the autoradiograph (*right panel*). A number of other nonfilaggrin or Am1 (data not shown) reactive bands were labeled with ^{32}P including a high molecular weight smear (*bar*) that is probably profilaggrin. The visualization of profilaggrin (> 200 kDa) on the Western blot (*left panel*) is masked by the very strong immunoreactive band above 100 kDa, which is the rabbit IgG reacting with the antirabbit secondary antibody.

peptide reacts with both antibodies it must contain both N-terminal and filaggrin sequences, and probably corresponds to an N-terminal processing intermediate consisting of the N-terminal A and B domains plus the truncated filaggrin unit, which is 457 amino acids in length with a predicted molecular weight of 50.3 kDa. The 28–30 kDa band, corresponding to the fully processed profilaggrin N-terminal peptide present in mouse skin, was not detected in either detached or adherent MEK cells grown in low calcium, or in cells grown in high calcium to induce differentiation (**Fig 4*A***).

An important aspect of profilaggrin processing to filaggrin is the removal of phosphate by serine/threonine phosphatases, which is necessary for filaggrin to associate with keratin filaments (Harding and Scott, 1983; Kam *et al*, 1993). Immunoprecipitation of ^{32}P-labeled MEK proteins and autoradiography showed that mouse profilaggrin was heavily phosphorylated *in vitro* (as is the case in epidermis) (Lonsdale-Eccles *et al*, 1982; Harding and Scott, 1983) whereas the 50 kDa and 90 kDa N-terminal peptides were not significantly phosphorylated (**Fig 4*B***). The other ^{32}P-labeled proteins observed in the 70–30 kDa range might be phosphoproteins that associate with the 50 kDa N-terminal peptide.

Filaggrin and profilaggrin N-terminal sequences specify cytoplasmic *versus* nuclear localization in transfected epithelial cells Previous results demonstrated that the human N-terminal peptide accumulates in nuclei of granular and transition cells of normal epidermis and in some parakeratotic skin disorders where nuclei persist in the stratum corneum (Ishida-Yamamoto *et al*, 1998; Presland and Dale, 2000). In contrast, filaggrin peptide sequences remain in the cytoplasm associated with keratin filaments. In order to examine the regulation of nuclear translocation we utilized transient transfections of multiple constructs into COS cells and keratinocytes. COS-7 cells expressing a cDNA construct encoding the N-terminus and the first truncated filaggrin unit (PFlag467proF; Presland *et al*, 1997) exhibited a diffuse and granular cytoplasmic localization (**Fig 5*A***). In contrast, when filaggrin sequences were absent from the expressed proteins, the human and mouse profilaggrin N-termini localized almost exclusively to the nuclei of transfected cells as detected by both GFP epifluorescence (**Fig 5*B–E***) and immunolabeling with N-terminal specific antibodies (data not shown). The nuclear morphology of transfected cells appeared similar to those of adjacent control cells, suggesting that expression of N-terminal peptides had no adverse effects on nuclear integrity.

Similar results were obtained when these constructs, i.e., mouse and human N-terminus containing or lacking filaggrin sequences, were transfected into REKs (data not shown).

Identification of functional nuclear localization sequences in the profilaggrin N-terminus Both human and mouse profilaggrin contain putative bNLSs in the B domain of the N-terminal peptide (**Fig 1*B***). This sequence usually consists of two groups of basic residues separated by an ≈10 residue spacer peptide of variable sequence (Dingwall and Laskey, 1986). The human profilaggrin bNLS identified by the program PSORT II lies between amino acids 121 and 137 (RKRPSSLERRNNRKGNK, **Fig 1*C***). To determine the functionality of this bNLS, a series of constructs was prepared in which this sequence was either mutated or deleted, and the effect on nuclear accumulation was examined. As shown in **Fig 6**, mutation of essential basic residues in the C-terminal end of this motif (RKGNK to LLGAA) (*panels C*, *D*) or deletion of the entire bNLS (*panels E*, *F*) prevented the specific

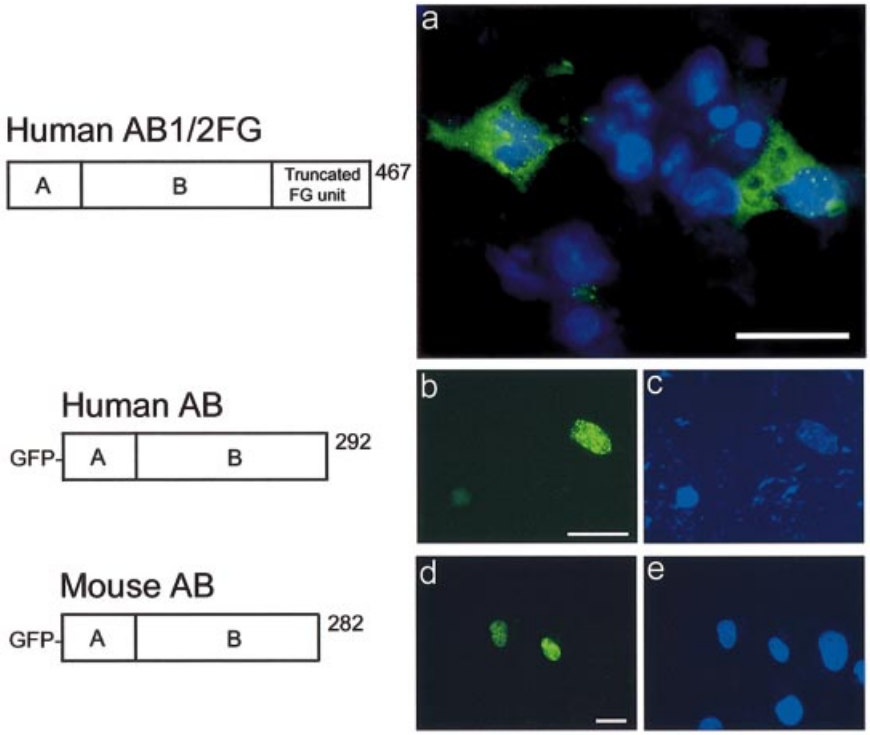

Figure 5. Cytoplasmic and nuclear distribution of expressed profilaggrin N-terminal peptides. COS-7 cells were transfected with the indicated constructs and fixed, and cellular distribution was examined by staining either with the human N-terminal B1 antibody (*A*) or by GFP autofluorescence (*B–E*). Part (*A*) shows that pFLAG467proF protein has a punctate, mostly cytoplasmic distribution (green, B1 antibody merged with blue DAPI counterstain, blue) whereas the GFP-tagged N-terminal human and mouse proteins (green, *B*, *D*) colocalized with the DAPI-stained nuclei (*C*, *E*) in expressing cells. A control GFP construct exhibited diffuse epifluorescence throughout the cytoplasm and nucleus as observed previously (**Fig 6*G***, ***H***; Kuechle *et al*, 1999). *Scale bars*: (*A*) 25 μm, (*B*)–(*E*) 10 μm.

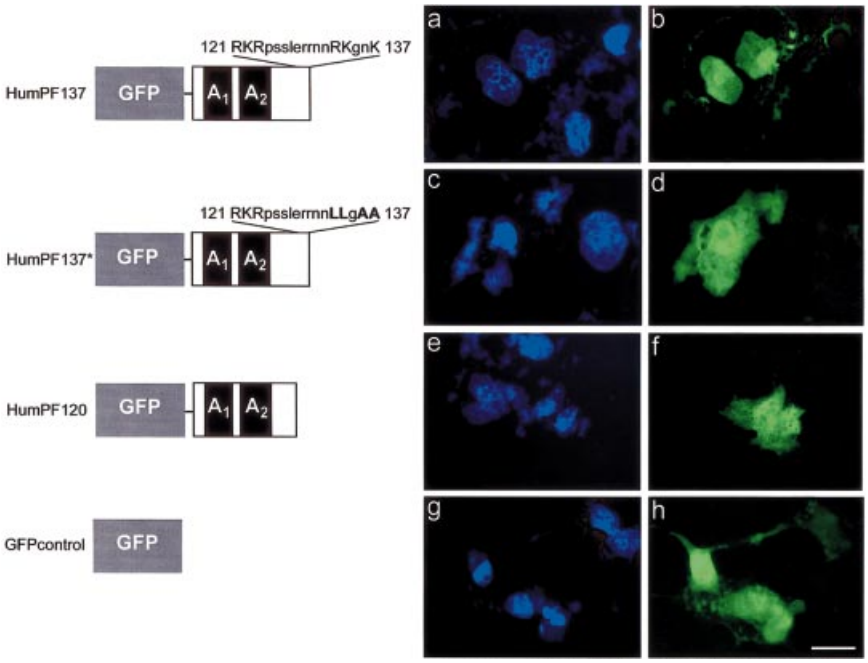

Figure 6. Identification of the nuclear localization sequence in the human profilaggrin N-terminus. The indicated GFP fusion constructs were transfected and analyzed by epifluorescence after either staining with DAPI to identify nuclei (*A*, *C*, *E*, *G*) or GFP fluorescence (*B*, *D*, *F*, *H*). Shown are cells expressing amino acids 1–137 terminating at the end of the bNLS (*A*, *B*); amino acids 1–137 with the C-terminus of the bNLS altered from RKGNK to LLGAA (*C*, *D*); and amino acids 1–120 in which the bNLS was deleted (*E*, *F*). Parts (*G*) and (*H*) show the parent GFP protein transfected into cells. Only in (*B*) is the profilaggrin–GFP restricted to the nucleus; note that mutation or deletion of the bNLS in human profilaggrin leads to loss of preferential nuclear accumulation of transfected protein. *Scale bar*: 10 μm.

nuclear localization of this peptide in COS-7 cells. These proteins with bNLS mutations or deletion exhibited a cytoplasmic and nuclear distribution pattern similar to that of the control GFP (*panels G, H*). Removal of sequences C-terminal to the bNLS of human profilaggrin, i.e., amino acids 138–293, did not measurably affect nuclear localization of the N-terminal peptide (compare **Fig 5*B***, ***C*** with **Fig 6*A***, ***B***). Similar results were obtained in REKs transfected with these same GFP–profilaggrin N-terminal constructs (data not shown).

The mouse profilaggrin N-terminal peptide contains four potential bNLSs in the B domain (**Fig 1*C***). To assess their function, deletion constructs were prepared that removed from 0 to 4 of the putative bNLSs. The results show that removal of NLS3 and NLS4 had little effect on nuclear localization of the fusion protein (**Fig 7*B***); however, removal of NLS2 or all four NLSs severely abrogated nuclear accumulation in transfected cells (**Fig 7*C–E***). These results show that NLS1 and NLS2 are essential for nuclear accumulation of the mouse profilaggrin N-terminal peptide, confirming that these motifs function as nuclear localization signals (**Table I**). The other two NLSs, NLS3 and NLS4, either are functionally redundant, i.e., are not required for nuclear targeting of the peptide, or do not function as NLSs.

In summary, these studies demonstrate that both the human and mouse profilaggrin N-terminal peptides contain functional bNLSs

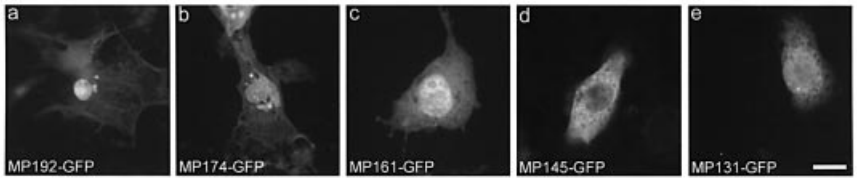

Figure 7. Identification of nuclear localization sequences in the mouse profilaggrin N-terminus. Mouse profilaggrin–GFP fusion constructs were transfected into COS-7 cells and analyzed by epifluorescence to determine intracellular distribution of GFP signal (*A–E*) relative to a DAPI counterstain to identify nuclei (not shown). Constructs were named according to the number of amino acids of mouse profilaggrin N-terminus they contain. MP192–GFP and MP174–GFP, containing four and two intact NLSs, respectively, are specifically localized to nuclei of transfected cells whereas other MP–GFP constructs show mostly a cytoplasmic distribution (summarized in **Table I**). *Scale bar*: 10 μm.

Table I. Summary of nuclear/cytoplasmic distribution of mouse profilaggrin–GFP constructs[a]

Construct	Contains	Nuclear	Cytoplasmic
MP192–GFP[b]	NLS1–4	++	+/–
MP174–GFP	NLS1, 2	++	+/–
MP161–GFP	NLS1	+	++
MP145–GFP	None	+/–	++
MP131–GFP	None	+/–	++

[a]Results summarize the intracellular (nuclear *versus* cytoplasmic) distribution of mouse profilaggrin N-terminus constructs that contain variable numbers of nuclear localization signals present in the mouse sequence (**Fig 1**). Data were derived from several experiments, including the results presented in **Fig 7**.

[b]Constructs were named according to the number of amino acids of mouse profilaggrin N-terminus they contain. MP, mouse profilaggrin.

and that these sequences are sufficient to account for the nuclear accumulation of these proteins *in vitro*.

DISCUSSION

The profilaggrin N-terminal peptide contains functional nuclear localization signals Profilaggrin undergoes proteolytic processing during epidermal terminal differentiation to generate two stable end-products, filaggrin and the N-terminal peptide. Whereas the function of filaggrin as a keratin binding and aggregating protein and as a likely source of hygroscopic amino acids in the stratum corneum has been extensively studied (Dale *et al*, 1978; Steinert *et al*, 1981; Manabe *et al*, 1991; Rawlings *et al*, 1994), the function of the released N-terminal peptide is unknown.

In this study we show that the cationic B domain of human and mouse profilaggrin contains functional nuclear localization sequences of the bipartite type, supporting a role for this peptide in nuclear events during epidermal terminal differentiation. In human profilaggrin, mutation or deletion of the bNLS prevented its nuclear translocation in transfected epithelial cells demonstrating that it functions as a nuclear targeting signal (**Fig 6**). These NLSs probably account for the observed accumulation of the processed human profilaggrin N-terminus in epidermal granular and transition cells (Ishida-Yamamoto *et al*, 1998; Presland and Dale, 2000). Mouse epidermis was more difficult to study because of the rapid transition from granular to cornified cells; however, the mouse N-terminus also localized to the nuclei of transfected epithelial cells (**Figs 5, 7**).

The profilaggrin N-terminal peptide is an S100-like calcium-binding protein (Presland *et al*, 1995) that can show either a cytoplasmic or nuclear localization depending on whether or not it is attached to filaggrin sequences, which is presumably a function of differentiation (see **Fig 8**). S100 proteins function by binding to target proteins in a calcium-regulated manner (reviewed in Donato, 1999). Like other S100 proteins, the profilaggrin N-terminal peptide undergoes a conformational change upon Ca^{2+} binding, suggesting that it might function like other S100 proteins by binding target proteins within the cytoplasm or nucleus (Presland *et al*, 1995). These downstream targets might include nuclear proteins such as proteases or nucleases involved in nuclear breakdown or cytoplasmic proteins that the profilaggrin N-terminal peptide transports to the nucleus. Alternatively, the N-terminal peptide might function as a DNA-binding protein consistent with the basic nature of the B domain, which shows some similarity to nucleic acid binding proteins such as histones. Other examples of EF-hand proteins that are present in both the nuclear and cytoplasmic compartments include the p53-regulated gene S100A2 (CaN19) (Deshpande *et al*, 2000), S100A6 (calcyclin) (Stradal and Gimona, 1999), and calmodulin (Deisseroth *et al*, 1998). S100A2, now classified as a tumor suppressor gene, is believed to function in the cellular proof-reading response to oxidative stress, whereas nuclear calmodulin functions to activate the transcription factor CREB via phosphorylation. The highly specific expression and release of the profilaggrin N-terminal peptide into the nuclei of epidermal cells coincident with terminal differentiation suggests a role for this peptide in nuclear events related to the normal desquamation process.

Processing of the mouse profilaggrin N-terminus occurs in two stages, and the products are associated with different subcellular compartments Our results suggest that profilaggrin N-terminal processing occurs in two steps, generating first a protein (50 kDa) that contains a truncated filaggrin unit and subsequently the free N-terminus (28–30 kDa). The enzyme that carries out this initial cleavage downstream of the truncated filaggrin unit might correspond to the protease that cleaves the YYY sequence of the mouse filaggrin linker (Resing *et al*, 1989; 1995a; 1995b), or a different protease. Only the initial 50 kDa processing intermediate was detected in cultured MEKs suggesting that the protease that releases the free N-terminus, e.g., furin or a related PC (Pearton *et al*, 2001) is not functional in cultured MEKs. The cytoplasmic localization of N-terminal filaggrin proteins (analogous to the 50 kDa profilaggrin peptide detected in cultured MEKs) can be accounted for by the strong affinity of filaggrin units for cytoplasmic keratin IFs (Dale *et al*, 1997; Kuechle *et al*, 1999; Presland *et al*, 2001).

Previously, we proposed that human profilaggrin undergoes N-terminal processing to release the free N-terminal peptide of 32 kDa (Presland *et al*, 1997). Similarly, in mouse epidermis only the free N-terminal peptide was detected (**Fig 4*A***). The absence of N-terminal peptide containing filaggrin sequences, i.e., the 50 kDa species, in mouse epidermis suggests that this processing intermediate is rapidly cleaved *in vivo* to generate the free N-terminal peptide.

This mechanism allows fine control of the two disparate functions of profilaggrin. Removal of the N-terminal filaggrin peptide would leave the polyfilaggrin region available for proteolytic processing while this N-terminal peptide is retained in the cytoplasm. Subsequent removal of the half-filaggrin (truncated) filaggrin domain frees the N-terminal domain, which is translo-

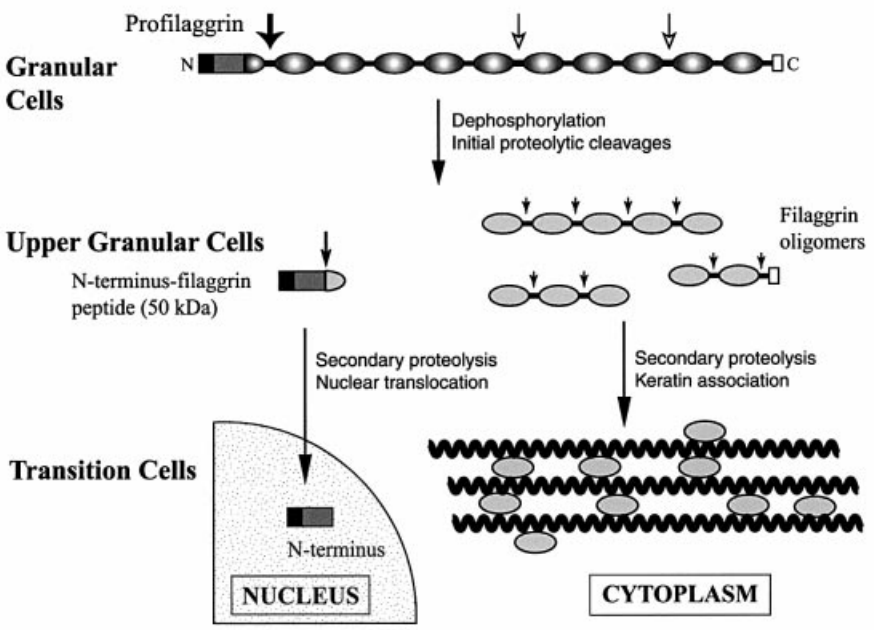

Figure 8. Model of profilaggrin processing and localization of proteolytic products during the granular to cornified cell transition. See text for details.

cated to the nucleus where we postulate it functions in nuclear events related to terminal differentiation.

A model for the control of profilaggrin processing and regulated subcellular localization of its two products Based on the findings reported here, as well as earlier studies, the following model is proposed for profilaggrin processing during the granular to cornified cell transition (**Fig 8**). Profilaggrin is synthesized in the epidermal granular layer where it is rapidly phosphorylated and accumulates in keratohyalin granules. Sequestration of this phosphoprotein in keratohyalin prevents premature association between filaggrin and keratin, which has deleterious effects on cell structure and viability *in vitro* (Dale *et al*, 1997; Kuechle *et al*, 1999; Presland *et al*, 2001).

As terminal differentiation occurs, the first N-terminal processing event occurs, releasing a peptide containing the N-terminal domain and the truncated (half-filaggrin) unit. This peptide corresponds to the abundant 50 kDa polypeptide seen in cultured MEKs grown in low Ca^{2+} (**Fig 4**). Cleavage of the N-terminal peptide from profilaggrin, coupled with dephosphorylation, may loosen the profilaggrin aggregate and allow greater access to further proteolytic enzymes, facilitating processing of the profilaggrin intermediates, i.e. the polyfilaggrin region, to filaggrin.

The first N-terminal processing event is possibly the first proteolytic event in profilaggrin processing and might occur shortly after initiation of profilaggrin processing in upper granular or "transition" cells. Subsequently, a calcium-dependent proteolytic cleavage occurs rapidly in transition cells (**Fig 8**) and results in release of the free N-terminus from the half-filaggrin sequence. The protease that carries out this second cleavage event may be either furin or a related PC enzyme (Pearton *et al*, 2001). The free N-terminal peptide then translocates to the nucleus. Immunohistochemical studies suggest that some N-terminal peptide is not transported to the nucleus, but rather is incorporated into the cornified envelope (Presland *et al*, 1997).

In contrast to the N-terminal peptide, the polyfilaggrin portion is processed to mature filaggrin units that bind to, and aggregate, the keratin IFs (**Fig 8**). These aggregated filaments form the dense macrofibrils of cornified cells and protect the keratins from proteolytic destruction (Manabe *et al*, 1991). In the upper cornified layers, filaggrin is finally processed to free amino acids including the compound pyrrolidone carboxylic acid derived from glutamine residues (Thulin and Walsh, 1995). These modified amino acids are thought to help maintain the osmotic balance in the outer cornified layers (Rawlings *et al*, 1994; Presland and Dale, 2000).

In summary, we present evidence for nuclear localization of the profilaggrin N-terminus, an event that occurs upon its removal from profilaggrin during terminal differentiation. The N-terminal processing of profilaggrin occurs in two steps, which enables keratinocytes to regulate the subcellular localization of its end-products, thus allowing cells to control profilaggrin processing and possibly other events in terminal differentiation where these end-products of profilaggrin proteolysis function.

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REFERENCES

Baden HP, Kubilus J: The growth and differentiation of cultured newborn rat keratinocytes. *J Invest Dermatol* 80:124–130, 1983

Brodersen DE, Etzerodt M, Madsen P, *et al:* EF-hands at atomic resolution: the structure of human psoriasin (S100A7) solved by MAD phasing. *Structure* 6:477–489, 1998

Dale BA, Holbrook KA, Steinert PM: Assembly of stratum corneum basic protein and keratin filaments in macrofibrils. *Nature* 276:729–731, 1978

Dale BA, Resing KA, Presland RB: Keratohyalin granule proteins. In: Leigh I, Lane B, Watt F, eds. *The Keratinocyte Handbook*. Cambridge: Cambridge University Press, 1994:pp 323–350

Dale BA, Presland RB, Lewis SP, *et al:* Transient expression of epidermal filaggrin in cultured cells causes collapse of intermediate filament networks with alteration of cell shape and nuclear integrity. *J Invest Dermatol* 108:179–187, 1997

Deisseroth K, Heist EK, Tsien RW: Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature* 392:198–202, 1998

Deshpande R, Woods TL, Fu J, *et al:* Biochemical characterization of S100A2 in human keratinocytes: subcellular localization, dimerization, and oxidative cross-linking. *J Invest Dermatol* 115:477–485, 2000

Di Cunto F, Topley G, Calautti E, *et al:* Inhibitory function of p21Cip1/WAF1 in

differentiation of primary mouse keratinocytes independent of cell cycle control. *Science* 280:1069–1072, 1998

Dingwall C, Laskey RA: Protein import into the cell nucleus. *Annu Rev Cell Biol* 2:367–390, 1986

Donato R: Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta* 1450:191–231, 1999

Fietz MJ, McLaughlan CJ, Campbell MT, Rogers GE: Analysis of the sheep trichohyalin gene: potential structural and calcium-binding roles of trichohyalin in the hair follicle. *J Cell Biol* 121:855–865, 1993

Hager B, Bickenbach JR, Fleckman P: Long-term culture of murine epidermal keratinocytes. *J Invest Dermatol* 112:971–976, 1999

Harding CR, Scott IR: Histidine-rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. *J Mol Biol* 170:651–673, 1983

Haydock PV, Blomquist C, Brumbaugh S, *et al:* Antisense profilaggrin RNA delays and decreases profilaggrin expression and alters *in vitro* differentiation of rat epidermal keratinocytes. *J Invest Dermatol* 101:118–126, 1993

Ishida-Yamamoto A, Iizuka H: Structural organization of cornified cell envelopes and alterations in inherited skin disorders. *Exp Dermatol* 7:1–10, 1998

Ishida-Yamamoto A, Takahashi H, Presland RB, *et al:* Translocation of profilaggrin N-terminal domain into keratinocyte nuclei with fragmented DNA in normal human skin and loricrin keratoderma. *Laboratory Invest* 78:1245–1253, 1998

Ishida-Yamamoto A, Kato H, Kiyama H, *et al:* Mutant loricrin is not crosslinked into the cornified cell envelope but is translocated into the nucleus in loricrin keratoderma. *J Invest Dermatol* 115:1088–1094, 2000

Kam E, Resing KA, Lim SK, Dale BA: Identification of rat epidermal profilaggrin phosphatase as a member of the protein phosphatase 2A family. *J Cell Sci* 106:219–226, 1993

Kilby PM, Van Eldik LJ, Roberts GC: The solution structure of the bovine S100B protein dimer in the calcium-free state. *Structure* 4:1041–1052, 1996

Koch PJ, de Viragh PA, Scharer E, *et al:* Lessons from loricrin-deficient mice: compensatory mechanisms maintaining skin barrier function in the absence of a major cornified envelope protein. *J Cell Biol* 151:389–400, 2000

Krieg P, Schuppler M, Koesters R, *et al:* Repetin (Rptn), a new member of the 'fused gene' subgroup within the S100 gene family encoding a murine epidermal differentiation protein. *Genomics* 43:339–348, 1997

Kuechle MK, Thulin CD, Presland RB, Dale BA: Profilaggrin requires both linker and filaggrin peptide sequences to form granules: implications for profilaggrin processing *in vivo*. *J Invest Dermatol* 112:843–852, 1999

Lee SC, Kim IG, Marekov LN, *et al:* The structure of human trichohyalin. Potential multiple roles as a functional EF-hand-like calcium-binding protein, a cornified cell envelope precursor, and an intermediate filament-associated (cross-linking) protein. *J Biol Chem* 268:12164–12176, 1993

Lonsdale-Eccles JD, Teller DC, Dale BA: Characterization of a phosphorylated form of the intermediate filament-aggregating protein filaggrin. *Biochemistry* 21:5940–5948, 1982

Makino T, Takaishi M, Morohashi M, Huh NH: Hornerin, a novel profilaggrin-like protein and differentiation-specific marker isolated from mouse skin. *J Biol Chem* 276:47445–47452, 2001

Manabe M, Sanchez M, Sun T-T, Dale BA: Interaction of filaggrin with keratin filaments during advanced stages of normal human epidermal differentiation and in ichthyosis vulgaris. *Differentiation* 48:43–50, 1991

Marenholz I, Volz A, Ziegler A, *et al:* Genetic analysis of the epidermal differentiation complex (EDC) on human chromosome 1q21: chromosomal orientation, new markers, and a 6-Mb YAC contig. *Genomics* 37:295–302, 1996

Markova NG, Marekov LN, Chipev CC, *et al:* Profilaggrin is a major epidermal calcium-binding protein. *Mol Cell Biol* 13:613–625, 1993

Mischke D, Korge BP, Marenholz I, *et al:* Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ('epidermal differentiation complex') on human chromosome 1q21. *J Invest Dermatol* 106:989–992, 1996

Missero C, Calautti E, Eckner R, *et al:* Involvement of the cell-cycle inhibitor Cip1/WAF1 and the E1A-associated p300 protein in terminal differentiation. *Proc Natl Acad Sci USA* 92:5451–5455, 1995

Nemes Z, Steinert PM: Bricks and mortar of the epidermal barrier. *Exp Mol Med* 31:5–19, 1999

Pearton DJ, Nirunsuksiri W, Rehemtulla A, *et al:* Proprotein convertase expression and localization in epidermis: evidence for multiple roles and substrates. *Exp Dermatol* 10:193–203, 2001

Presland RB, Dale BA: Epithelial structural proteins of the skin and oral cavity: function in health and disease. *Crit Rev Oral Biol Medical* 11:383–408, 2000

Presland RB, Haydock PV, Fleckman P, *et al:* Characterization of the human epidermal profilaggrin gene. Genomic organization and identification of an S-100-like calcium binding domain at the amino terminus. *J Biol Chem* 267:23772–23781, 1992

Presland RB, Bassuk JA, Kimball JR, Dale BA: Characterization of two distinct calcium-binding sites in the amino-terminus of human profilaggrin. *J Invest Dermatol* 104:218–223, 1995

Presland RB, Kimball JR, Kautsky MB, *et al:* Evidence for specific proteolytic cleavage of the N-terminal domain of human profilaggrin during epidermal differentiation. *J Invest Dermatol* 108:170–178, 1997

Presland RB, Boggess D, Lewis SP, *et al:* Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris. *J Invest Dermatol* 115:1072–1081, 2000

Presland RB, Kuechle MK, Lewis SP, *et al:* Regulated expression of human filaggrin in keratinocytes results in cytoskeletal disruption, loss of cell–cell adhesion, and cell cycle arrest. *Exp Cell Res* 270:199–213, 2001

Rawlings AV, Scott IR, Harding CR, Bowser PA: Stratum corneum moisturization at the molecular level. *J Invest Dermatol* 103:731–740, 1994

Resing KA, Walsh KA, Haugen-Scofield J, Dale BA: Identification of proteolytic cleavage sites in the conversion of profilaggrin to filaggrin in mammalian epidermis. *J Biol Chem* 264:1837–1845, 1989

Resing KA, Al-Alawi N, Blomquist C, *et al:* Independent regulation of two cytoplasmic processing stages of the intermediate filament-associated protein filaggrin and role of Ca^{2+} in the second stage. *J Biol Chem* 268:25139–25145, 1993a

Resing KA, Johnson RS, Walsh KA: Characterization of protease processing sites during conversion of rat profilaggrin to filaggrin. *Biochemistry* 32:10036–10045, 1993b

Resing KA, Johnson RS, Walsh KA: Mass spectrometric analysis of 21 phosphorylation sites in the internal repeat of rat profilaggrin, precursor of an intermediate filament associated protein. *Biochemistry* 34:9477–9487, 1995a

Resing KA, Thulin C, Whiting K, *et al:* Characterization of profilaggrin endoproteinase 1. A regulated cytoplasmic endoproteinase of epidermis. *J Biol Chem* 270:28193–28198, 1995b

Rothnagel JA, Mehrel T, Idler WW, *et al:* The gene for mouse epidermal filaggrin precursor. Its partial characterization, expression, and sequence of a repeating filaggrin unit. *J Biol Chem* 262:15643–15648, 1987

Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning, a Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, 1989

Sastry M, Ketchem RR, Crescenzi O, *et al:* The three-dimensional structure of Ca(2+)-bound calcyclin: implications for Ca(2+)-signal transduction by S100 proteins. *Structure* 6:223–231, 1998

Smith SP, Shaw GS: A change-in-hand mechanism for S100 signalling. *Biochem Cell Biol* 76:324–333, 1998

Song HJ, Poy G, Darwiche N, *et al:* Mouse Sprr2 genes: a clustered family of genes showing differential expression in epithelial tissues. *Genomics* 55:28–42, 1999

South AP, Cabral A, Ives JH, *et al:* Human epidermal differentiation complex in a single 2.5 Mbp long continuum of overlapping DNA cloned in bacteria integrating physical and transcript maps. *J Invest Dermatol* 112:910–918, 1999

Steinert PM, Cantieri JS, Teller DC, Lonsdale-Eccles JD, Dale BA: Characterization of a class of cationic proteins that specifically interact with intermediate filaments. *Proc Natl Acad Sci USA* 78:4097–4101, 1981

Suga Y, Jarnik M, Attar PS, *et al:* Transgenic mice expressing a mutant form of loricrin reveal the molecular basis of the skin diseases, Vohwinkel syndrome and progressive symmetric erythrokeratoderma. *J Cell Biol* 151:401–412, 2000

Thulin CD, Walsh KA: Identification of the amino terminus of human filaggrin using differential LC/MS techniques: implications for profilaggrin processing. *Biochemistry* 34:8687–8692, 1995

Yamazaki M, Ishidoh K, Suga Y, *et al:* Cytoplasmic processing of human profilaggrin by active mu-calpain. *Biochem Biophys Res Commun* 235:652–656, 1997